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Award Number: DAMD17-00-1-0560

TITLE: Characterization of the Hen as a Model for Human Ovarian Cancer

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REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Characterization of the Hen as a Model for Human Ovarian Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0560

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Investigation of basic factors involved in malignant transformation of the ovary has been hampered by the lack of an appropriate animal model. The overall hypothesis of this project is that the hen is an excellent model for human ovarian epithelial cell cancer. We will take a three-pronged approach in this project. First, we will compare differences in spontaneous incidence between two strains of hens as they age and examine for pathological ovarian changes that may indicate site of origin of the tumors. We have not yet compared the incidence of tumors in the two strains of hens because we were not able to obtain 2-year old hens at the start of the experiment. We have begun to histologically analyze the ovarian surface epithelium (OSE) as well as ovary proper for pre-tumor lesions. Our second approach was to evaluate possible differences between the strains in response to reproductive manipulations highly correlated to incidence in women. Because we did not have access to 2 year old hens at the initiation of the experiment, we have not yet begun experiments to manipulate the incidence of adenocarcinoma. These experiments will be initiated in the coming year. Our third approach was to examine potential differences in cell signaling that may underlie the different incidence between the strains. We have made the most progress on this task with respect to validating and characterizing our culture system for OSE cells. We have determined that significant quantities of hen OSE cells can be obtained and can be cultured in two commercially available types of media (CM) or (MCDB) for at least 10 days. The cultured OSE cells are positive for cytokeratin and negative for vimentin. In combination with Hoechst staining, these results indicate that the cultures are not contaminated with fibroblasts.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Ovarian cancer, hen, animal model	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Investigation of basic factors involved in malignant transformation of the ovary has been hampered by the lack of an appropriate animal model. Most animals do not spontaneously develop ovarian cancer. This may be related to the fact that the usual condition of most wild and domestic animals is pregnancy and/or lactation. The exception is the domestic chicken, which has been demonstrated by several investigators to spontaneously develop ovarian cancer (Campbell, 1951; Wilson, 1958; Fredrickson, 1987). In this respect, as well as the fact that the chicken is a persistent ovulator (laying breeds ovulate almost daily), the chicken is similar to modern day women. That is, most women have 10-20 years of monthly ovulations prior to one or two pregnancies, with a subsequent 10-20 years of ovulations prior to menopause. The overall hypothesis of our DOD supported project is that the hen is an excellent model for human ovarian epithelial cell cancer. We will take a three-pronged approach in this project. First, we will compare differences in spontaneous incidence between the C and K strains of hens as they age and examine for pathological ovarian changes that may indicate site of origin of the tumors. Second, we will evaluate possible differences between the strains in response to reproductive manipulations highly correlated to incidence in women; and third, we will examine potential differences in cell signaling that may underlie the different incidence between the strains. It would be possible to conduct these experiments with a commercial strain of hens. We anticipate however, that use of the related C and K strains with different incidences of the disease, will provide a powerful tool that may reveal a potential marker of ovarian cancer.

BODY

Our start date for the work was somewhat delayed because a Research Associate was not hired until December 1, 2000.

Task 1. To characterize the incidence of spontaneous ovarian adenocarcinoma in 3-5 year old hens of the C and K strains and document histological changes in the ovary that may precede tumor formation (months 1-30)

The overall goal of this task is to document the incidence of adenocarcinoma in hens of the C and K strain during their third, fourth and fifth year. In addition, we hope that frequent histological analysis of the ovary may reveal pre-cancer lesions. As mentioned previously, incidence data for these strains was determined at approximately 2 years and we and others have seen a dramatic increase as hens age. Use of the two strains (C and K) will indicate whether the difference in incidence between the two strains in younger hens is maintained with age. We have not previously documented the incidence in hens of the C and K strains at 3-5 years of age because of the expense of keeping the hens for that length of time. This characterization of incidence in these 2 genetic strains with age is critical to be able to compare the 2 strains with the hope of identifying a possible marker.

At this point we have not yet begun to compare the incidence of tumors in the two strains of hens at 6 month intervals. We were not able to obtain 2-year old hens at the start of the experiment, so we had to wait for them to mature. At this point we have approximately 100 each of C and K strain hens at 1 year of age and 100 of each at 2 years old. Within the coming year, we will begin to systematically examine the hens to identify and classify tumors and pre-tumors. We have been maintaining laying records on all hens as an indication of ovulation rate. In addition, we have removed samples of blood from C and K strain hens to assess hormone levels but these have not yet been analyzed. We have begun to histologically analyze the ovarian surface epithelium as well as ovary proper for pre-tumor lesions. Figure 1 depicts a section of hen ovary stained with hematoxylin and eosin.

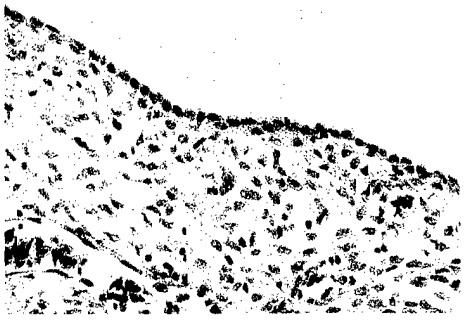
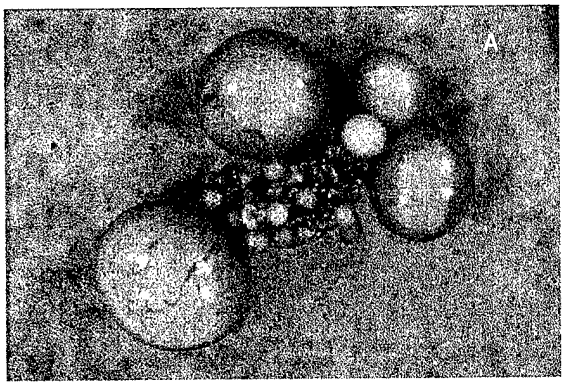


Figure 1: Paraffin section of the hen's ovary stained with hematoxylin and eosin. A single layer of cuboidal cells is seen on the surface of the ovary with underlying stromal cells (100 X magnification).



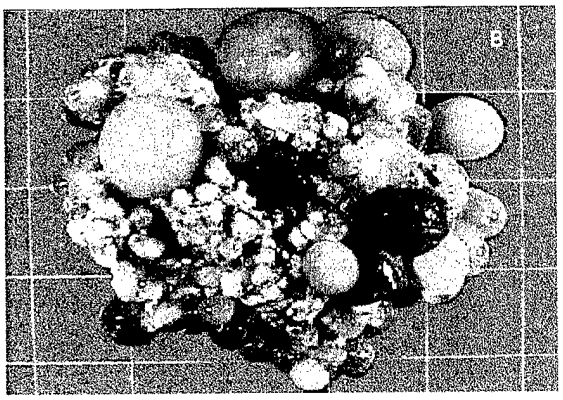
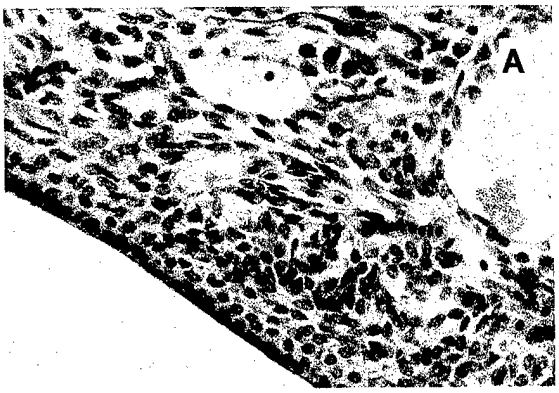


Figure 2: **Panel A: Normal ovary of a hen. Panel B: Ovary of a hen with ovarian adenocarcinoma. The ovary is characterized by the "cauliflower-like" protrusions as well as several hemorrhagic follicles and cystic-like structures.**



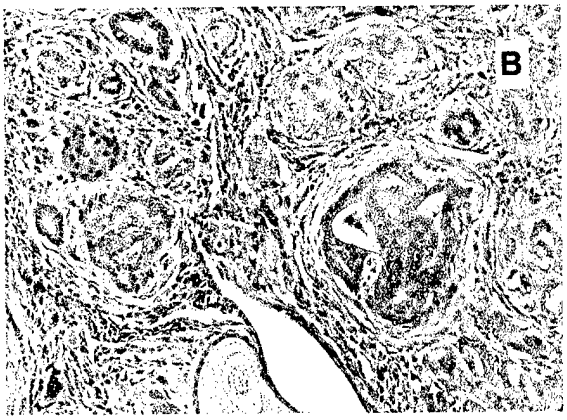


Figure 3: **Paraffin sections of the hen's ovary stained with hematoxylin and eosin.**

Panel A: Normal ovary.

Panel B: Ovary from a hen with ovarian adenocarcinoma. The tumor is characterized with cystically dilated glands.

A picture of the normal ovary as well as an ovary with advanced ovarian adenocarcinoma is shown in Figure 2. Representative sections through a normal ovary and an ovary with adenocarcinoma is shown in Figure 3. We have utilized a histology service on campus to make histological sections of normal ovaries as well as those with frank adenocarcinoma in order to become increasingly informed about the malignant process. We are ready to begin the examination of the C and K strain hens at regular intervals to document pre-malignant changes that may be occurring. We have been consulting with an avian pathologist at the University of California at Davis (Dr. H.L. Shivaprasad). He has examined tissue samples and tumors for us and given us a diagnosis.

Task 2. To manipulate the incidence of ovarian adenocarcinoma in the C and K strain of hens to test the effect of ovulation rate on a different genetic background.

Because we did not have access to 2 year old hens at the initiation of the experiment, we have not yet begun experiments to manipulate the incidence of adenocarcinoma. These experiments will be initiated in the coming year.

Task 3. To characterize the activity of the Activin/Smad signal transduction system in cell signaling in the normal ovarian epithelial layer and tumors from the C and K strain hens.

We have made the most progress on this task with respect to validating and characterizing our culture system for the culture of ovarian surface epithelial cells. More than 90% of ovarian cancers are believed to arise from the single layer of epithelial cells that covers the ovarian surface (Figure 1). In order to begin the experiments for this task, it was necessary to further characterize our culture system for the ovarian surface epithelial cells. Therefore, we began with three main objectives: 1) to determine an optimal method of recovering OSE cells from the ovary of the hen; 2) to optimize culture conditions for OSE cells; and 3) to characterize the OSE cells in terms of expression of the intermediate filaments cytokeratin and vimentin.

In Study 1, Babcock hens (n=9) were euthanized and large yellow follicles (F3, F4 and F5) were collected. We began these studies with a commercial strain of hens because we wanted to first validate our system and not use our limited special strains. Ovarian surface epithelial cells were collected by washing, mildly vortexing, or scraping the follicles with a rubber policeman. Cells were washed and suspended in CM (DMEM:M199 + 10% FCS) or MCDB (MCDB:M199 + 10% FCS) and plated in duplicate in 8 well LabTek Chamber Slides[â]. Cells were fixed on days 3 and 10 and examined for relative cell growth and the expression of the intermediate filaments cytokeratin and vimentin. Cell nuclei were stained with Hoechst 33258 to visualize all cells. The study was replicated three times. We observed no significant difference in the relative number of OSE cells cultured in the two media (data not shown). The relative number of OSE cells

recovered from the surface of the hen's ovary was significantly affected by the method used to collect them (Figure 4). Vortexing or scraping the ovarian surface resulted in more cells at 3 and 10 days of culture than washing the ovary.

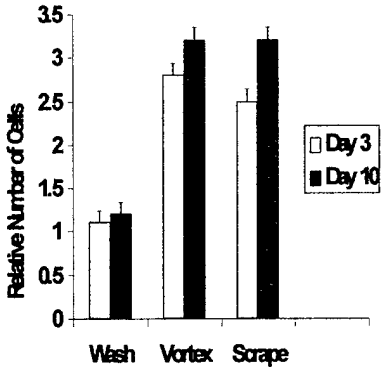


Figure 4: The relative cell growth of OSE cells as affected by the method of recovery from the ovarian surface ($P<0.01$).

In Figure 5, we show a representative sample of cultured ovarian surface epithelial cells stained with the Hoechst stain. We used this technique to demonstrate that our cultures were not contaminated with fibroblasts, a common type of contamination. We stained our cultures with Hoechst as well as with the cytokeratin antibody. We reasoned that we should see the nuclear stain of Hoechst in all cells and anticipated that the epithelial cells would also exhibit cytoplasmic staining positive for cytokeratin. This analysis was positive in that all cells in the culture were positive for cytokeratin and negative for vimentin. In addition, however, we realized that endothelial cells are also positive for cytokeratin. We stained our cultures with low-density lipoprotein, acetylated DiI complex which has been demonstrated to metabolically label endothelial cells (Voyta et al., 1984). This evaluation indicated that endothelial cells were present. Fortunately, endothelial cells have a different morphology from the cultured surface epithelial cells, so they can be distinguished. We are currently modifying our culture protocol to limit endothelial cell contamination.

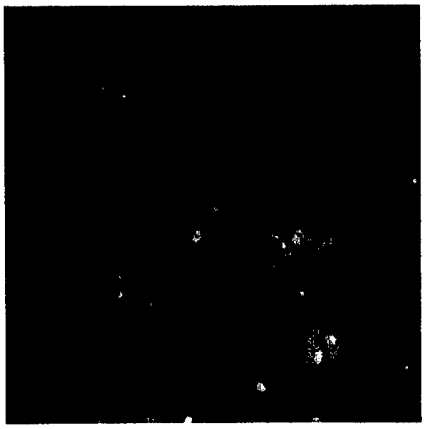


Figure 5: Cultured OSE cells dual stained with Hoechst stain and cytokeratin antibody

In Study 2, Babcock hens (n=3) were euthanized and large yellow follicles (F3, F4 and F5) were collected and scraped with a rubber policeman. Cells were washed and suspended in CM or MCDB and plated in 96 well plates. The relative number of cells was determined on days 3 and 10 after plating by using a colorimetric method (CellTiter 96â Aqueous One Solution Cell Proliferation Assay). Data are expressed (Figure 6) as the fold increase over control cultures 16 hours after plating. The study was replicated three times.

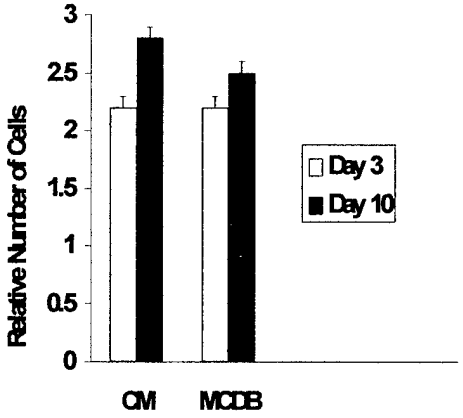


Figure 6: The relative cell growth of OSE cells cultured in CM or MCDB media for 3 or 10 days.

In Figure 7, a field of cultured chicken ovarian epithelial cells is pictured. These cells exhibit typical epithelial cell morphology. These cells were cultured for 10 days in CM. They were uniformly positive for cytokeratin and negative for low-density lipoprotein, acetylated, DiI.

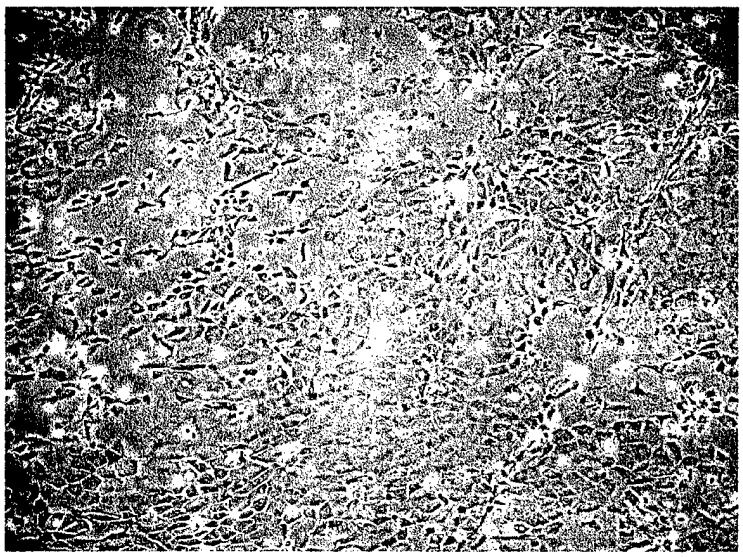


Figure 7: Micrograph of hen OSE cells cultured for 10 days (280 X magnification).

KEY RESEARCH ACCOMPLISHMENTS

1. We have isolated the surface epithelial layer of the hen and histologically examined the ovarian stroma in normal hens and hens with adenocarcinoma.

2. We have determined that significant quantities of ovarian surface epithelial cells can be obtained by scraping or vortexing ovarian follicles from the hen.

3. These cells can be cultured in two commercially available types of media (CM) or (MCDB) for at least 10 days.

4. The cultured OSE cells are positive for the intermediate filament cytokeratin and negative for vimentin. In combination with Hoechst staining, these results indicate that the cultures are not contaminated with fibroblasts.

REPORTABLE OUTCOMES

1) Giles, J.R., C. DeLeonardis and P.A. Johnson. The isolation and primary culture of ovarian surface epithelium cells from the hen: a model for human ovarian cancer. Biology of Reproduction 64(Suppl. 1):316, 2001. (Abstr.)

2) Presentation of these data by Dr. Giles at the annual meeting of the Society for the Study of Reproduction, Ottawa, Ontario, Canada, 2001.

3) Invitation for Dr. Johnson to present these data at the Ovarian Workshop, Baltimore, July, 2002.

CONCLUSIONS

This project is important because the hen spontaneously develops ovarian adenocarcinoma and therefore, questions related to etiology can be examined. This work is innovative because although previous workers have characterized ovarian adenocarcinoma in the hen, they have not attempted to manipulate the incidence nor studied the ovarian epithelial cell layer. In addition, the use of two related genetic strains which differ in spontaneous incidence of ovarian cancer may reveal an important difference between the two strains that could underlie the differential susceptibility to ovarian cancer.

Our initial studies were directed at comparing ovarian cytology in normal hens and those with ovarian adenocarcinoma. The lack of sufficiently aged birds at the initiation of the project made us delay our studies aimed at influencing the incidence of adenocarcinoma in hens. These

studies should commence in the coming year. Finally, the isolation and culture of large quantities of pure chicken OSE has proved to be a significant task. We are currently validating an explant method to eliminate possible contamination of endothelial cells. At this stage, we are quite confident of the purity and characteristics of the chicken OSE and will soon initiate additional studies (under task 3) requiring large quantities of pure OSE.

The main cause of the lethality of ovarian cancer is the fact that it is usually diagnosed at an advanced stage. The availability of an animal model which spontaneously develops ovarian cancer (unlike most other animal models) would enhance the chance of finding a marker for early diagnosis. Knowledge about the etiology of ovarian cancer may help in the design of more optimal treatments. In addition, an animal model would permit the testing of pharmaceuticals that may decrease the growth of this cancer. Characterization of the two genetic strains may permit the identification of potential tumor markers.

REFERENCES

Campbell, J.G. Some unusual gonadal tumours of the fowl. Brit. J. of Cancer 5:69-82, 1951.

Fredrickson, T.N. Ovarian tumors of the hen. Environmental Health Perspectives 73:35-51, 1987.

Voyta, J.C., D.P. Via, C.E. Butterfield and B.R. Zetter. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low-density lipoprotein. J.of Cell Biology 99:2034-2040, 1984.

Wilson, J.E. Adeno-carcinomata in hens kept in a constant environment. Poultry Sci. 37:1253(abs.), 1958.

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THE ISOLATION AND PRIMARY CULTURE OF OVARIAN SURFACE EPITHELIUM CELLS FROM THE HEN-A MODEL FOR HUMAN OVARIAN CANCER

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Ovarian cancer is the most fatal gynecological malignancy in women although little information is available about the development due in part to the lack of an animal model. It is thought that most ovarian cancers originate from the ovarian surface epithelium (OSE). We are fortunate to have two strains of White Leghorn hens which have different incidences of spontaneous ovarian adenocarcinoma. The goal of this project is to develop a method of obtaining OSE cells from the ovary of the hen and to optimize conditions for culture. We utilized the previously described explant and scrape method as well as a brief vortex procedure to obtain OSE cells. Hens (n=10) were euthanized and the ovaries recovered. The explant procedure required collecting samples of small yellow (SYF) and large white (LWF) follicles and placing these directly into culture. The scrape method involved lightly scraping the surface of the larger follicles (F1-F5), SYF, LWF and the general surface of the ovary. The vortex method consisted of briefly vortexing ovarian tissue in medium prior to placing the cell suspension into culture. Medium was a 1:1 blend of DMEM and Medium 199, 15% FCS, 1% glutamine, antibiotics and antimycotic. Cultures were incubated under a humid atmosphere of 5% CO2 in air at 37 degrees C. The explant method resulted in mixed populations of epithelial- and fibroblast-like cells. The scrape method provided a more homogeneous population of epithelial-like cells and furthermore, scraping the general surface of the ovary was faster and resulted in larger quantities of cells than scraping SYF and LWF. Data on larger follicles were inconclusive. The vortex procedure similarly resulted in large quantities of quite pure epithelial-like cells. Fibroblast-like cells observed in these cultures were often in clumps and could be removed manually. Enzymatic passage of any epithelial-like cells did not result in confluent dishes of cells with a similar phenotype.
Immunohistochemical staining of samples of the primary cultures demonstrated that the epithelial-like cells were positive for cytokeratin and negative for

vimentin. These techniques can now be utilized to provide large quantities of OSE cells from the hen model and these cells may provide insights into the early events leading to ovarian cancer in humans. Supported by DAMD17-00-1-0560.

Keywords:

ovarian cancer, ovarian surface epithelium, hen